UT-A Urea Transporter Protein Expressed in Liver: Uptigation by Uremia

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Abstract. In perfused rat liver, there is phloretin-inhibitable urea efflux, but whether it is mediated by the kidney UT-A urea transporter family is unknown. To determine whether cultured HepG2 cells transport urea, thiourea influx was measured. HepG2 cells had a thiourea influx rate of 1739 ± 156 nmol/g protein per min; influx was inhibited 46% by phloretin and 32% by thionicotinamide. Western analysis of HepG2 cell lysate using an antibody to UT-A1, UT-A2, and UT-A4 revealed two protein bands: 49 and 36 kD. The same bands were detected in cultured rat hepatocytes, freshly isolated rat hepatocytes, and in liver from rat, mouse, and chimpanzee. Both bands were present when analyzed by native gel electrophoresis, and deglycosylation of rat liver lysate had no effect on either band. Differential centrifugation of rat liver lysate showed that the 49-kD protein is in the membrane fraction and the 36-kD protein is in the cytoplasm. To determine whether the abundance of these UT-A proteins varies in vivo, rats were made uremic by 5/6 nephrectomy. The 49-kD protein was significantly increased 5.5-fold in livers from uremic rats compared to pair-fed control rats. It is concluded that phloretin-inhibitable urea flux in liver may occur via a 49-kD protein that is specifically detected by a UT-A antibody. Uremia increases the abundance of this 49-kD UT-A protein in rat liver in vivo.

Urea is the main end product of protein metabolism. It is synthesized in the liver and subsequently excreted in the urine. Since urea is a small molecule, it is often thought to be freely permeable across cell membranes. However, urea is a highly polar molecule and has a low permeability across lipid bilayers (1). Physiologic findings in erythrocytes and kidney inner medullary collecting ducts (IMCD) show that urea transport occurs by facilitated (or carrier-mediated) pathways (reviewed in references 2 and 3).

In the past 5 yr, cDNA for facilitated urea transporters (UT) have been cloned from kidney (UT-A) (4–9) and erythrocytes (UT-B) (10–13). There are at least four members of the UT-A gene family: UT-A1, -A2, -A3, and -A4 (4–9). These isoforms are thought to originate from a common gene (UT-A) by alternative splicing (4,9). UT-A1 is the predominant kidney isoform and is expressed in the apical plasma membrane and cytoplasm of the terminal IMCD (14). Urea transport by UT-A1 is stimulated by vasopressin in Xenopus oocytes (4). A polyclonal antibody to the carboxy terminus of UT-A1 detects 97- and 117-kD proteins in the rat inner medulla (14–16). However, this antibody also detects UT-A2 (45 to 50 kD) and UT-A4 (approximately 37 kD, in vitro translated protein), since they share the same carboxy-terminal sequence as UT-A1 (9). Both UT-A2 and UT-A4 mRNA are expressed in rat outer and inner medulla (9).

In both kidney and erythrocytes, facilitated urea transport can be inhibited by phloretin (17–21). The only other mammalian tissue that has phloretin-inhibitable urea transport is the liver (22–24), suggesting that liver cells express a urea transporter protein. Liver cells may need a urea transporter to accelerate urea efflux following ureagenesis (22,25).

In perfused rat liver and freshly isolated hepatocytes, Effros and colleagues demonstrated phloretin-inhibitable urea efflux (22); however, whether it is mediated by a protein from the UT-A urea transporter family is unknown. Therefore, the goals of this study were to: (1) verify that liver cells express phloretin-inhibitable urea transport by measuring thiourea influx into cultured HepG2 (human hepatoblastoma) cells; (2) determine whether a UT-A protein is expressed in liver; and (3) determine whether the abundance of UT-A protein(s) is altered in livers from uremic rats since these animals have a pathophysiologic alteration in urea production.

Materials and Methods

Animals/Tissue Preparation

Male Sprague Dawley rats weighing approximately 90 g (initial weight) were sacrificed, and their kidneys, liver, lungs, brain, and colon were removed. These tissues were homogenized in isolation buffer (10 mM triethanolamine, 250 mM sucrose, 1 μg/ml leupeptin, 0.1 mg/ml phenylmethylsulfonyl fluoride, pH 7.6, 0.025 to 0.1 g
tissue per ml isolation buffer) (15,26). Concentrated sodium dodecyl sulfate (SDS) was added to the homogenized samples to achieve a final concentration of 1%, after which samples were sheared with a 28-gauge insulin syringe and centrifuged for 15 min at 14,000 × g. Protein was determined in the supernatant fractions using the BioRad DC protein assay kit (BioRad, Richmond, CA). For cellular localization, tissue was homogenized in isolation buffer, then centrifuged at 200,000 × g for 1 h to yield a membrane pellet and cytosolic supernatant (14). Western blots of the membrane and cytosolic proteins were probed with polyclonal antibodies to UT-A and NKCC1/BSC2 (generous gift of Dr. Christian Lytle, University of California at Riverside). To induce uremia, rats underwent 5/6 nephrectomy, were fed 40% protein, and drank one-quarter normal saline (27,28). Control rats underwent sham operation and were given the same food and drink. Blood was drawn for chemical analysis at the time of sacrifice and tissues were collected as listed above.

Cell Culture

HepG2 (American Type Culture Collection, CRL-10741) cells were cultured on multwell plates in minimal essential medium (Cellgro, Herndon, VA) supplemented with 10% fetal bovine serum, penicillin, and streptomycin (Pen/Strep, Life Technologies, Grand Island, NY), 1% nonessential amino acids, and 1 mM sodium pyruvate (Cellgro) at 37°C in 5% CO₂. Fresh medium was added every 3 d. Clone 9 (American Type Culture Collection, CRL-1439) cells (normal rat hepatocytes) were cultured in Ham’s F12K medium supplemented with 10% fetal bovine serum, 2 mM glutamine, and Pen/Strep.

Hepatocyte Preparation

Hepatocytes were kindly provided by Dr. Susan Voss (Emory University) and were isolated as described by Voss et al. (29).

Western Blot/Protein Analysis

Proteins (10 μg/lane) were separated on 10% SDS-polyacrylamide gels, then transferred to a polyvinylidene difluoride membrane. Membranes were probed with affinity-purified polyclonal anti-UT-A1 (prepared against the carboxy-terminal portion of the protein [15], 3.8 mg/ml used at 1:5000 for Western blot), and immunoreactive proteins were visualized by enhanced chemiluminescence (Amersham, Arlington Heights, IL) (15,26). Autoradiograms were scanned using the BioRad Gel Doc 1000 digital imaging densitometer. Scanned bands were quantified using the system’s Multi-Analyst version 1.0.1 software. For antibody competition studies, primary antibody was preadsorbed against the carboxy-terminal portion of the protein [15], 3.8 mg/ml used at 1:5000 for Western blot), and immunoreactive proteins were visualized by enhanced chemiluminescence (Amersham, Arlington Heights, IL) (15,26). Autoradiograms were scanned using the BioRad Gel Doc 1000 digital imaging densitometer. Scanned bands were quantified using the system’s Multi-Analyst version 1.0.1 software.

Statistical Analyses

All data are presented as mean ± SEM and n is the number of rats. t test was used to test for statistical significance. The criterion for statistical significance was P < 0.05. Unpaired statistical analysis was used, except for protocols in which the rats were pair-fed, where a paired statistical analysis was used and n was the number of rat pairs.

For the flux data, the net cpm/mg protein versus the elapsed time were fit to a single exponential. The initial slope of the fit line divided by the specific activity was the calculated flux in nmol/g protein per min. The standard error of each flux value was calculated by dividing the standard error of the slope by the specific activity. The flux was linear for 5 min for the thiourea influx measurements. Statistical significance was determined using the unpaired two-tailed t test.

Results

Urea Influx

We tested for phloretin-inhibitable urea transport by measuring influx of the urea analog 14C-thiourea (32). Cultured HepG2 cells have a high rate of thiourea influx (1800 nmol 14C-thiourea/g protein per min) (Figure 1). Phloretin (100 μM) significantly inhibited thiourea influx by 46%. Thionicotininamide (2 mM [31]), when present, was added to the thiourea influx buffer. Exchanges were staggered, resulting in radiolabel incubations of: 0, 1, 2, 4, 5, and 20 min. The influx was terminated by removing the thiourea solution and rapidly washing three times with ice-cold stopping solution (in mM): 143.9 NaCl, 1.8 CaCl₂, 0.81 MgCl₂, 5.3 KCl, and 5 Hepes, pH 7.4. Cells were lysed with 25 μl/well of 0.1% Triton X-100 and incubated for 30 min at room temperature, followed by the addition of 500 μl of 25 mM NaOH and 0.5% sodium deoxycholate. Protein determinations were performed on 100 μl of the well contents. The radiolabel content was determined in 450 μl by liquid scintillation counting. All fluxes were performed in duplicate.

Figure 1. Thiourea influx in cultured HepG2 cells. The graph presents a typical single result from an influx assay with averaged results shown in Figure 2. Each point represents the thiourea content of a single well of cells after incubation with radiolabel for a fixed amount of time. The two points at each time are duplicate wells. The flux is determined as a rate of movement of radiolabel into the cells fit to a single exponential equation: y = yoe⁻kt.
namide (2 mM, a structural analog of thiourea) also significantly inhibited thiourea influx by 32% (Figure 2). Thiourea influx in HepG2 cells was higher than in freshly isolated rat hepatocytes (1100 nmol 14C-thiourea/g protein per min; data not shown); phloretin (100 μM) significantly inhibited thiourea influx by 32%.

Western Analysis
To determine the abundance of UT-A protein(s) present in liver, we used a polyclonal antibody that was prepared to the carboxy terminus of UT-A1 (14,15,26). This antibody should identify UT-A1, UT-A2, and UT-A4 due to their shared carboxy-terminal sequence (9) as diagrammed in Figure 3. Two protein bands were detected at 49 and 36 kD (Figure 4). The 97- and 117-kD UT-A1 proteins normally seen in kidney inner medulla were not observed in liver. When other rat tissues were probed for UT-A, only brain showed a protein band at 52 kD (Figure 4).

To determine whether the finding of UT-A proteins in liver was unique to the rat, we probed for the presence of UT-A proteins in: (1) livers from mouse and chimpanzee; (2) isolated hepatocytes from normal rats; and (3) cultured HepG2 cells and clone 9 cells (normal rat liver cells). Figure 5 shows that both the 49- and 36-kD proteins are present in all species tested and in all forms of liver cells tested. However, the relative abundance of the 49- and 36-kD proteins varied between liver tissue and the cultured cell lines. In liver tissue from all three species tested, the 36-kD protein was much more abundant than the 49-kD protein. However, in the cultured liver cell lines, the 49-kD protein was more abundant than the 36-kD protein.

Competition studies were performed to verify that both UT-A protein bands were being specifically recognized and were not the result of some nonspecific interaction. The antibody was preadsorbed with the immunizing peptide against which the antibody was prepared. Figure 6 shows a comparison of Western blots of proteins from kidney inner medulla (IM) or liver (L) probed with normal antibody and preadsorbed antibody. In rat inner medulla, neither of the UT-A1 protein bands (97 or 117 kD) was detected with the preadsorbed antibody.
consistent with previous results (15,16). In rat liver, the 49- and 36-kD proteins were detected by the antibody. However, neither band was detected by the preadsorbed antibody, indicating specific recognition of both the 49- and 36-kD UT-A proteins in liver (and the 97- and 117-kD UT-A1 proteins in kidney).

Probing Western blots with preimmune serum (from the same rabbit that was used to make the antibody) also showed no bands at the molecular weights of the UT-A proteins in either kidney or liver.

To determine whether the 49- and 36-kD proteins found in liver were subunits of the larger UT-A1 protein, liver lysate was prepared under nondenaturing conditions and proteins were resolved by native (nondenaturing) polyacrylamide gel electrophoresis (PAGE). The Western blot of this gel (Figure 7A) shows that these isoforms are two distinct proteins. To determine whether these two protein bands represent one protein with different glycosylation states, rat liver lysate was treated with peptide \( N \)-glycosidase F (PNGase F) or \( O \)-glycanase. The size of the liver UT-A proteins was unchanged by PNGase F treatment, suggesting that they are not \( N \)-glycosylated proteins (Figure 7B). As a control, we treated kidney inner medulla lysate with PNGase F and probed for aquaporin-2 (AQP-2; the AQP-2 antibody was a generous gift of Dr. H. William Harris, Harvard University, Boston, MA). When probed for AQP-2, Western analysis (Figure 7C) shows a protein band at 29 kD and a smear from 35 to 45 kD (33); PNGase F treatment eliminated the 35- to 45-kD smear (34). The size of the liver UT-A proteins was also unchanged by \( O \)-glycanase treatment, suggesting that they are not \( O \)-glycosylated proteins (Figure 7D). As a positive control, fetuin was treated with \( O \)-glycanase and showed the expected decrease in protein size (Figure 7E).

To determine the cellular location of these proteins, liver lysates were subjected to centrifugation at 200,000 × g for 1 h to prepare cytosolic and membrane fractions (14). These fractions were then analyzed for UT-A content by Western blot.
analysis. Figure 8 (top panel) shows that the 49-kD protein is found exclusively in the membrane fraction, whereas the 36-kD protein is present in cytoplasm and absent from the membrane. Membrane fraction purity was verified by the presence of the Na\(^+-\)K\(^+-\)2Cl\(^-\) cotransporter (NKCC1/BSC2) protein (Figure 8, bottom panel) only in the whole liver lysate and membrane fractions.

Pathophysiologic Regulation

Having established that facilitated urea transport and a UT-A protein isoform are present in liver, we carried out an initial study of its pathophysiologic significance by testing whether the abundance of this protein was altered by a pathologic condition associated with changes in urea metabolism: uremia. Normal rats were subjected to 5/6 nephrectomy and fed a 40% protein diet for 8 d to produce uremia; control rats underwent sham operation (27,28). The nephrectomized rats had significantly higher blood urea nitrogen values than the sham-operated control rats (Table 1). However, there was little change in the serum albumin, total protein, and electrolyte levels, suggesting that the uremic rats were not malnourished or dehydrated.

The abundance of the 49-kD UT-A protein was significantly increased in livers from the uremic rats (Figure 9A). There is also a suggestion of a concomitant decrease in the abundance of the 36-kD protein; however, despite optimization of electrophoresis and film exposure conditions, this failed to reach statistical significance (\(P = 0.08\)). Densitometric analysis (Figure 9B) revealed a 5.42-fold increase in the 49-kD band (\(P < 0.05\)) in livers from the uremic rats compared to the sham-operated control rats.

Because the nephrectomized rats tend to eat less as they become uremic, we repeated the preceding protocol using a second series of five pairs of rats in which the uremic and sham-operated rats were pair-fed. The nephrectomized rats had significantly higher blood urea nitrogen values than the sham-
operated control rats, and the blood chemistries were similar between the non-pair-fed rats and the pair-fed rats (Table 1). We again found a significant increase in the abundance of the 49-kD protein without a significant change in the abundance of the 36-kD protein (data not shown).

Table 1. Serum values in sham-operated and uremic rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Study 1: Rats Fed ad Libitum</th>
<th>Study 2: Rats Pair-Fed</th>
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<tbody>
<tr>
<td></td>
<td>(n = 4)</td>
<td>(n = 6)</td>
</tr>
<tr>
<td>Urea nitrogen (mg/dl)</td>
<td>28 ± 2</td>
<td>123 ± 22(b)</td>
</tr>
<tr>
<td>Creatinine</td>
<td>0.6 ± 0.1</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>Sodium (mEq/L)</td>
<td>137 ± 3</td>
<td>155 ± 11</td>
</tr>
<tr>
<td>Chloride (mEq/L)</td>
<td>97 ± 1</td>
<td>116 ± 9</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>3.7 ± 0.2</td>
<td>2.6 ± 0.5</td>
</tr>
<tr>
<td>Total protein (g/dl)</td>
<td>6.1 ± 0.1</td>
<td>4.9 ± 0.8</td>
</tr>
<tr>
<td>SO Rats</td>
<td>30 ± 5</td>
<td>104 ± 24(b)</td>
</tr>
<tr>
<td>Uremic Rats</td>
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<td>1.1 ± 0.1(b)</td>
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<tr>
<td></td>
<td>126 ± 11</td>
<td>132 ± 1</td>
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<td></td>
<td>86 ± 2</td>
<td>94 ± 2(b)</td>
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<tr>
<td></td>
<td>2.8 ± 0.1</td>
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<tr>
<td></td>
<td>5.1 ± 0.1</td>
<td>5.7 ± 0.2</td>
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\(a\) Results are given as mean ± SEM. SO, sham-operated.
\(b\) \(P\) < 0.05 uremic versus control.

Discussion

The major results of our study are that: (1) phloretin-inhibitable, facilitated urea transport is present in cultured human hepatoblastoma (HepG2) cells; (2) 49- and 36-kD UT-A proteins are present in liver from several mammalian species and cultured cells; (3) the 49-kD protein is present in the cell membrane, whereas the 36-kD protein is present in the cytoplasm; and (4) the 49-kD UT-A protein is upregulated in livers from uremic rats. The 49- and 36-kD UT-A proteins are specifically recognized by our antibody to UT-A1, UT-A2, and UT-A4 (9,15). These three UT-A isoforms run at different sizes on SDS-PAGE: UT-A1, fully glycosylated, runs at 97 and 117 kD (16); UT-A2 runs at 45 to 50 kD (5,6,9); and UT-A4 runs at 37 kD (9). Based on the size of the proteins, the 49-kD liver protein may be UT-A2. Although the size of the 36-kD protein might suggest that it is UT-A4, we consider this unlikely since the 36-kD protein is cytosolic. This suggests that the 36-kD protein is unlikely to contribute to transcellular urea transport and may represent a transition protein of some kind. Further investigation will be required to better characterize this 36-kD protein. It is also possible that the liver expresses unique UT-A isoforms that have not yet been cloned.

We also detected a seemingly unique UT-A protein isoform of 52 kD in the brain. Previous studies of brain demonstrated expression of UT-B mRNA, but not UT-A (5,7,11,13,35). The 52-kD protein that we detected immunologically is unlikely to be UT-B since our antibody to UT-A does not recognize UT-B protein in erythrocytes (unpublished observation).

Because the liver is the principal site of ureagenesis, it is logical that hepatocytes may need a urea transporter, and a few previous studies provided evidence for a liver urea transporter. Effros and colleagues demonstrated phloretin-inhibitable urea efflux from perfused rat livers and freshly isolated rat hepatocytes (22). Von Dahl and Haussinger also demonstrated this urea efflux in perfused rat livers (23). Walsh and colleagues observed phloretin-sensitive urea transport in hepatocytes from the gulf toadfish (25). You et al., using a cDNA probe to UT-A2, identified a 4-kb mRNA in rabbit liver that is consistent with the size of UT-A1 (5). Recently, Smith and Wright cloned a facilitated urea transporter cDNA from the kidney of Squalus acanthias that is most homologous to UT-A2 and that
hybridizes to a 3-kb mRNA in liver from *Squalus acanthias* by Northern analysis (36). These studies suggest that at least one function for a hepatic urea transporter is to facilitate the rapid transport of urea out of hepatocytes (following ureagenesis) and into the extracellular space (22,23,25). Our studies of thiourea influx in cultured HepG2 cells confirm these previous findings and extend them by directly demonstrating phloretin-inhibitable urea transport in cultured human liver cells.

For the present study, we chose a phloretin concentration that was similar to what Effros and colleagues used in their study (22). Although phloretin is the best studied inhibitor of urea transport, it does not inhibit urea transport completely, either in the perfused rat terminal IMCD (17) or when UT-A2 is expressed in *Xenopus* oocytes (4–6,37); the percent inhibition that we observed with 100 μM phloretin is consistent with the percent inhibition observed in these studies. We do not know what pathway mediates the phloretin-insensitive component of thiourea flux; however, a portion of it may simply be the bilayer permeability.

**Uremic Rats**

To begin to investigate the pathophysiologic role of urea transporters in liver, we studied the abundance of the 49- and 36-kD UT-A proteins in livers from rats made uremic by 5/6 nephrectomy (27,28). Perez and colleagues and Klim and colleagues showed that chronic uremia is accompanied by increases in hepatic urea production (38,39). We showed that UT-A1 protein abundance is altered in a pathophysiologic condition, uncontrolled diabetes mellitus (26). In the renal inner medulla of rats with uncontrolled diabetes mellitus, we found that UT-A1 protein abundance was decreased due to an increase in glucocorticoids (26). Thus, regulation of UT-A protein abundance is one mechanism by which urea transport is regulated *in vivo*. In the present study, we found that the 49-kD UT-A membrane protein was upregulated in livers from uremic rats. This result suggests that upregulation of this urea transporter may be an adaptive response to uremia that allows the hepatocytes to excrete urea.

Urea is thought to be a marker of uremia rather than a toxin per se. However, urea is synthesized from NH₄⁺ and HCO₃⁻. Since excessive blood levels of NH₄⁺ are quite toxic, we speculate that upregulation of UT-A proteins may allow hepatocytes to increase urea production (38,39) to reduce the accumulation of NH₄⁺ (40).

**Summary**

The present studies demonstrate that mammalian liver cells express phloretin-inhibitable transport of the urea analog thiourea. They also demonstrate that mammalian liver expresses a 49-kD UT-A urea transporter protein that is located in the cell membrane and whose abundance is upregulated in livers from uremic rats.

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**References**


